# Natural or Synthetic RNA Delivery: A Stoichiometric Comparison of Extracellular Vesicles and Synthetic Nanoparticles

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# Supporting Information

# Methods

### *Cell Culture*

HEK293T, MDA-MB-231 and A431 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with L-Glutamine (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Streptomycin and penicillin was added to all cell culture medium at 100 µg/ml and 100 u/ml (Gibco) respectively. Stable HEK293T reporter cell lines expressing the CROSS-FIRE reporter construct and Cas9 and sgRNA expressing donor MDA-MB-231 and A431 lines were prepared and cultured with selection antibiotics as previously described[1].

### *EV Isolation*

Targeting-sgRNA or non-targeting control sgRNA expressing MDA-MB-231 and A431 cells were cultured in T175 flasks. Cells were seeded in 6 batches of 20 flasks at varying densities so that each batch would reach approximately 90% confluency on 6 consecutive days in order to isolate EVs on 6 consecutive days for addition experiments. At 90% confluency, the FBS containing culture medium was aspirated and cells were washed with 10ml PBS. 20ml of serum free OptiMEM (Gibco) was then added and cells were cultured for a further 24 hours. The conditioned OptiMEM was then spun for 5 minutes at 4°C at 300 x g to remove detached cells followed by 2000 x g for 15 minutes at 4°C to remove dead cells and cell debris. Conditioned medium was then filtered using a 0.45µm bottle top filter and concentrated to approximately 10ml using tangential flow filtration with a peristaltic pump attached to a Minimate 100 kDa Omega Membrane cassette (Pall Corporation). This medium was further concentrated to 1ml using a 100 kDa Amicon Ultra-15 Centrifugal filter (Merck). EVs were then isolated using size exclusion chromatography using a Tricorn 10/300 column with Sepharose 4 Fast Flow resin attached to an AKTA Start or AKTA Pure chromatography system (all GE Healthcare Life Sciences). Fractions containing the EV peak were then pooled, 0.45µm syringe filtered for sterility and concentrated to approximately 180µl using a 100 kDa Amicon Ultra-15 Centrifugal filter.

### *LNP Production*

DLin-MC3-DMA containing LNPs were composed of DLin-MC3-DMA/Cholesterol/DSPC/PEG-DMG at a molar ratio of 50/38.5/10/1.5. LNPs were produced by microfluidic mixing in a NanoAssemblr Benchtop device (Precision Nanosystems, Vancouver, BC, Canada). Prior to production, lipids were diluted to a total lipid concentration of 5mM in pure ethanol while RNA was diluted in a 25mM acetate buffer (pH4). LNPs were produced with an N/P ratio of 12 at a flow of 6ml/minute with a flow rate ratio of 3:1 aqueous to lipid phase. LNPs were then dialysed into PBS overnight using 20kDa G2 dialysis cassettes (ThermoFisher). After dialysis, LNPs were sterilized by membrane filtration using 0.45µm syringe membrane filters. Final encapsulated RNA concentration was determined using a Quant-iT Ribogreen assay (ThermoFisher) according to manufacturer's instructions which confirmed that encapsulation efficiency was close to 100%.

### *Size and Zeta potential measurement*

Dynamic Light Scattering (DLS) was performed to measure the hydrodynamic radius of EVs and DLin-MC3-DMA-LNPs using a Nano S Zetasizer instrument (Malvern). Samples were diluted in DPBS and light scattering was measured at an angle of 173 at 25°C for 10s and repeated at least 10 times. Zeta potential measurement was performed on samples using a Nano Z Zetasizer device on samples diluted in 0.1x PBS.

### *Western Blotting*

Cells and EV samples were lysed in RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were spun at max speed at 4°C for 5 minutes to pellet precipitates. Protein concentrations were determined using a MicroBCA protein assay according to Manufacturer's protocol (ThermoFisher Scientific). Sample input was normalised by loading an equal weight of total protein per sample. Samples were mixed with sample loading buffer with 100μM DTT, or without DTT when blotting for tetraspanins. Samples were run on a 4-12% gradient Bis-Tris polyacrylamide gel (Thermo Fisher Scientific) before blotting onto Immobilon-FL polyvinylidine difluoride membranes (Millipore). Membranes were then blocked overnight at 4°C by incubation in blocking buffer composed of Odyssey Blocking Buffer (LI-COR Biosciences) mixed 1:1 with Tris-Buffered Saline, 0.1% tween (TBS-T). Membranes were then probed overnight at 4°C using primary antibodies diluted 1:500 in blocking buffer. These antibodies were ALIX 1:500 (ThermoFisher Scientific, MA1-83977), Calnexin 1:500 (GeneTex, GTX101676), CD9 1:500(Abcam, ab92726), CD63 1:500 (Abcam, ab8219) and COX IV (Abcam, ab33985). After primary antibody incubation, the blots were washed 3 times for 10 minutes at room temperature in TBS-T. Secondary antibodies were applied overnight at 4°C at a 1:7500 dilution in blocking buffer. The secondary antibodies used were anti-rabbit IgG conjugated to AlexaFluor680 (ThermoFisher Scientific, A-21076) or anti-mouse IgG conjugated to IR dye 800CW. Blots were then washed 3 times for 10 minutes at room temperature in TBS-T and scanned using an Odyssey Infrared Imager (LI-COR Biosciences) at 700nm and 800nM.

### *Nanoparticle tracking analysis*

EV and LNP particle concentrations were determined using a Nanosight S500 nanoparticle analyzer (Malvern Instruments) equipped with a 405nm laser. Samples were diluted in PBS to a concentration that gave between 30 to 100 tracks per frame. Videos were acquired with a camera level of 16. EVs were analyzed with a detection threshold of 6 while LNPs were analzyed with a detection threshold of 4, due to their smaller size. NTA Software version 3.3 was used for video analysis.

### *RT-qPCR*

Firstly, EV samples of unknown targeting-sgRNA concentration were diluted to 250ul in PBS. Standard curve samples of known targeting-sgRNA concentration were prepared in 250ul of PBS and were treated identically to EV samples throughout the entire process. Then, 750ul of Trizol LS (Life Technologies) was added to all samples. To allow normalisation for extraction efficiency, an equal quantity of non-targeting spike-in sgRNA was applied to all samples at this point. RNA was then isolated according to the manufacturer's protocol using GlycoBlue coprecipitant (Thermo Fisher Scientific). RNA pellets were resuspended in 20ul of nuclease free water and cDNA synthesis was performed on 10ul RNA suspension using a SuperScript 4 kit (Thermo Fisher Scientific), RNasin Ribonuclease inhibitor (Promega) and 2 pmol of sgRNA reverse primer which was specific for both targeting sgRNA and the non-targeting spike-in sgRNA. The resulting cDNA was diluted 1:5 in nuclease free water before qPCR analysis using iQ SYBR Green Supermix (Bio-Rad) in a CFX96 Real-Time PCR Detection System (Bio-Rad). To normalise for extraction efficiency, spike-in RNA Ct values were subtracted from targeting-RNA values. Using these normalised Ct values and starting dilution factor, the sgRNA concentration of EV samples was interpolated from the normalised standard curve Ct values using Graphpad Prism 8.0.1 software.

### *EV Addition experiments*

HEK293T CROSS-FIRE reporter cells were seeded in a volume of 200μl in a 96-well plate. EVs were then isolated from targeting-sgRNA or non-targeting control sgRNA expressing MDA-MB-231 and A431 cells. Particle concentration was determined using NTA after each isolation and the particle

concentrations of targeting and non-targeting EVs were normalised before addition. EVs were applied on 6 consecutive days in a high dose volume of 40µl or a low dose volume of 10µl. Medium was replaced before each addition and cell confluency was maintained between 30-100% throughout the experiment. After the last EV addition reporter cells were incubated for a further 24 hours to allow GFP expression. Reporter cells were then analysed using an EVOS FL Cell Imaging System (Thermo Fisher Scientific) and measured using a Fortessa (BD Biosciences) flow cytometer.

### *DLin-MC3-DMA-LNP Titration*

DLin-MC3-DMA LNPs were produced as described using targeting-sgRNA as cargo. After production, dialysis and filtration, final RNA concentration was determined using a Quant-iT Ribogreen assay (ThermoFisher) according to manufacturer's instructions. LNPs were diluted to so that upon addition to 200µl culture volume, the final concentration would be 10nM. A 1:10 dilution series of this LNP stock was prepared so that LNPs could be titrated onto HEK293T CROSS-FIRE reporter cells at a wide range of sgRNA concentrations. Cells were dosed with LNPs and treated in an identical manner to that of EV addition experiments.

### *Spike-in LNP Titration*

DLin-MC3-DMA LNPs were produced as described using scaffold-sgRNA as cargo. 6 different batches were produced with targeting sgRNA spiked in to achieve targeting to scaffold sgRNA ratios ranging from 1:325 to 1:32500000. After production, dialysis and filtration, final total RNA concentration was determined using a Quant-iT Ribogreen assay (ThermoFisher) according to manufacturer's instructions. LNPs were diluted to a 16.25nM stock so that upon addition of 50µl stock to 200µl culture volume, the final total RNA concentration would be 3.25nM. This gave final targeting-sgRNA concentrations ranging from 0.1fM to 10,000 fM. Cells were dosed with LNPs and treated in an identical manner to that of EV addition experiments.

### *In vitro transfection reagent titration*

HEK293T CROSS-FIRE reporter cells were transfected with targeting sgRNA at a concentration of 10nM using Lipofectamine RNAiMax and TransIT-2020 according to manufacturer's instructions. Reporter cells were transfected with 25 kDa linear polyethylenimine (PEI) by diluting 3ul of 1mg/ml 25kDa PEI stock in 50µl of OptiMEM. Targeting sgRNA was diluted in a separate tube of 50µl of OptiMEM and mixed with the PEI dilution and incubated for 30 minutes at room temperature to allow polyplex formation. The transfection mixes used to achieve a concentration of 10nM were serially diluted 1:10 in order to achieve the same final targeting sgRNA concentrations used in DLin-MC3-DMA-LNP titrations. Cells were transfected according to the same schedule as EV addition experiments.

### *Quantification of NP uptake*

MDA-MB-231-EVs and A431-EVs were labelled with MemGlow$^{TM}$560 according to manufacturer's instructions. DLin-DMA-MC3-LNPs were produced as described with the addition of 0.2% 18:1 Liss Rhod PE (Avanti Polar Lipids). HEK293T cells were seeded at the same density used in EV addition experiments and spike-in LNP titrations in CellStar 96-well cell culture black µClear bottom TC-treated microplates (Greiner-Bio). Cells were treated for 2, 4 or 24 hours with a similar dose of fluorescent particles as used in EV addition experiments and spike-in LNP titrations. Prior to measurement, medium was aspirated and cells were washed in PBS. Cells which had been treated with NPs were then lysed in 1x RIPA buffer. A standard curve was produced by lysing a series of wells containing cells which had not been treated with NPs in 1x RIPA. A 1:1 dilution series standard curve starting at 100% of the dose applied to the cells at the 2, 4 and 24 hour time points was then added to this lysed cell mixture. Fluorescent signal was then measured at excitation = 561, emission = 601 on a

Spectramax™ plate reader. The percentage of total dose that had been taken up by cells could then be interpolated from the standard curve.

### *Confocal Microscopy*

MDA-MB-231-EVs and A431-EVs were labelled with MemGlow™560 according to manufacturer's instructions. DLin-DMA-MC3-LNPs were produced as described with the addition of 0.2% 18:1 Liss Rhod PE (Avanti Polar Lipids). HEK293T cells were seeded at the same density used in EV addition experiments and spike-in LNP titrations in CellStar 96-well cell culture black µClear bottom TC-treated microplates (Greiner-Bio). Cells were treated for 2, 4 or 24 hours with a similar dose of fluorescent particles as used in EV addition experiments and spike-in LNP titrations. 1 µg/ml Hoechst 33342 (ThermoFisher Scientific) was then added to the culture medium and a Yokogawa C7000 confocal microscope was used to produce confocal images at 40x magnification using the following filter settings: Hoechst: emission = 405 nm, power 30; acquisition =BP445/45, Exposure time = 200 ms. MemGlow™560 and Liss Rhod: emission = 561 nm, power 30; acquisition =BP600/37, Exposure time = 200ms.

### *FACS Analysis*

After treatment with EVs or synthetic transfection reagents, CROSS-FIRE reporter cells were trypsinised and resuspended in DMEM, 10% FBS. Cells were then transferred to a round bottom 96 well plate and pelleted by spinning at 300g for 5 minutes at 4°C. Cells were then resuspended in 200µl FACS buffer consisting of PBS, 1% FBS including 1µg/ml DAPI as a live dead stain. Cells were then analysed using a Fortessa or Canto (BD Biosciences) flow cytometer. FACs analysis was performed using FlowJo v10 software and gating was performed as shown in Figure S2.

### *Statistical Analysis*

All statistical analysis was performed using Graphpad prism software V8.01. EV addition experiments were analysed using ANOVA with a post-hoc Tukey's test. The minimal effective dose of DLin-MC3-DMA-LNPs, Spike-in DLin-MC3-DMA-LNPs and *in vitro* transfection reagents was determined in the same way using ANOVA with a post-hoc Tukey's test.

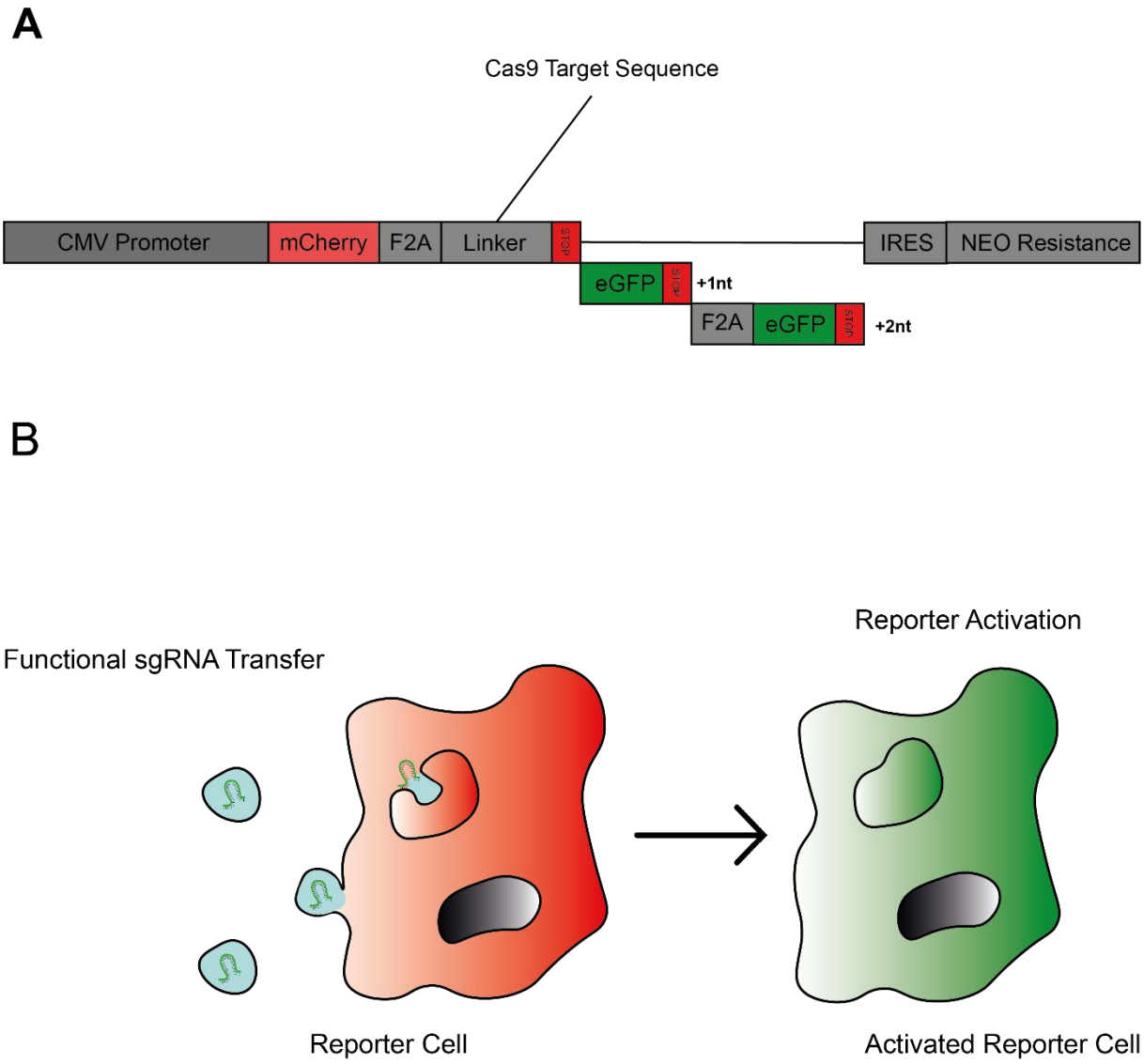


**Figure S1 – The CROSS-FIRE reporter system.**

The reporter construct is expressed under a CMV promoter. The first component is mCherry which allows identification of reporter cells. This is followed by a linker sequence which is specifically targeted by sgRNA bound to Cas9. Upon Cas9-mediated cleavage of the linker sequence, the cut is repaired through a process of non-homologous end joining. This process can introduce frameshift mutations which cause readthrough of the downstream stop codon and brings eGFP open reading frames, which are either 1 or 2 nucleotides out of frame, into frame. This results in the permanent expression of eGFP. F2A self-cleaving domains are placed between each fluorescent protein (A). This system allows the detection of functional sgRNA transfer to reporter cells which express the reporter construct and Cas9. In the absence of sgRNA, reporter cells express only mCherry. Upon functional delivery of sgRNA, Cas9 is guided to the reporter linker sequence and GFP expression is induced (B)[1].

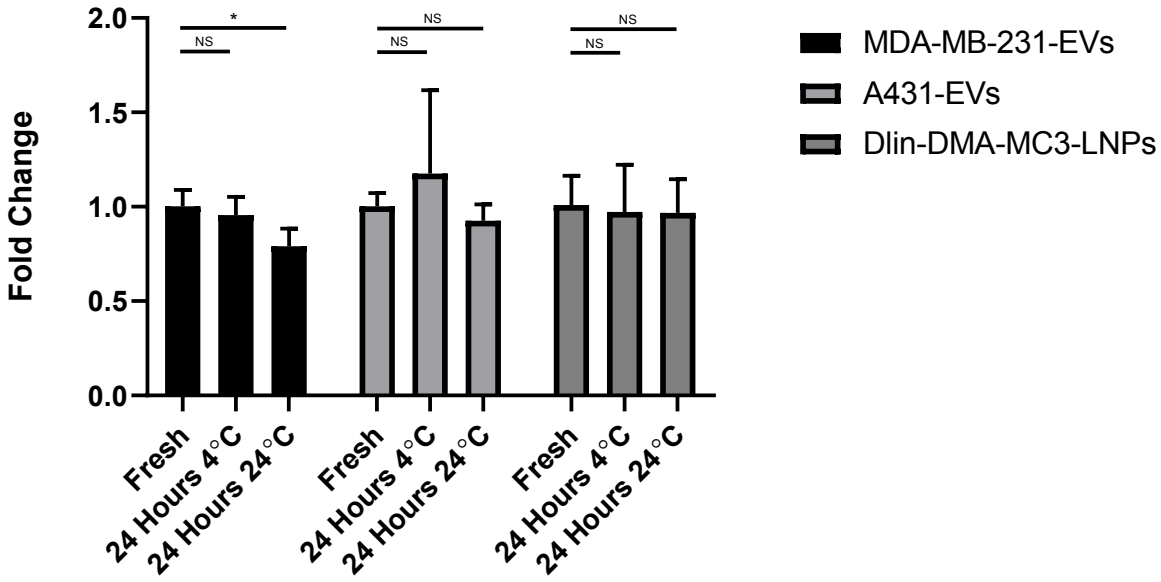


**Figure S2 – MDA-MB-231-EV, A431-EV and DLin-DMA-MC3-LNP associated sgRNA is stable.**

RT-qPCR analysis of targeting sgRNA in MDA-MB-231-EV, A431-EV and DLin-DMA-MC3-LNP samples freshly after isolation, after incubation at 24 hours of incubation at 4°C and 24 hours of incubation at 37°C. Fold changes were calculated relative to the average Ct value of the fresh condition of each NP type. N=4 technical replicates, Tukey's multiple comparison test, *=$p<0.05$.

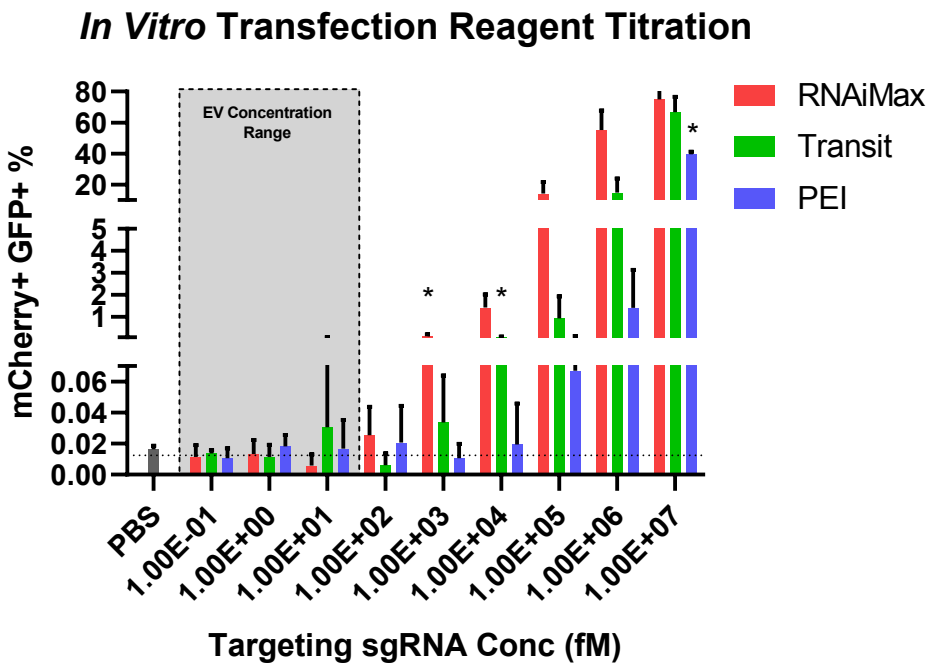


**Figure S3 - *in vitro* transfection reagent titration.**

Flow cytometry analysis of Stoplight+ spCas9+ HEK293T reporter cells after 6 consecutive daily transfections of targeting sgRNA using the typical in vitro transfection reagents RNAiMax, TransitIT-2020 and 25 kDa linear PEI at a range of 1E+07 fM to 1E-01 fM. Tukey's multiple comparison test, *=p<0.05, **=p<0.01 versus vehicle.

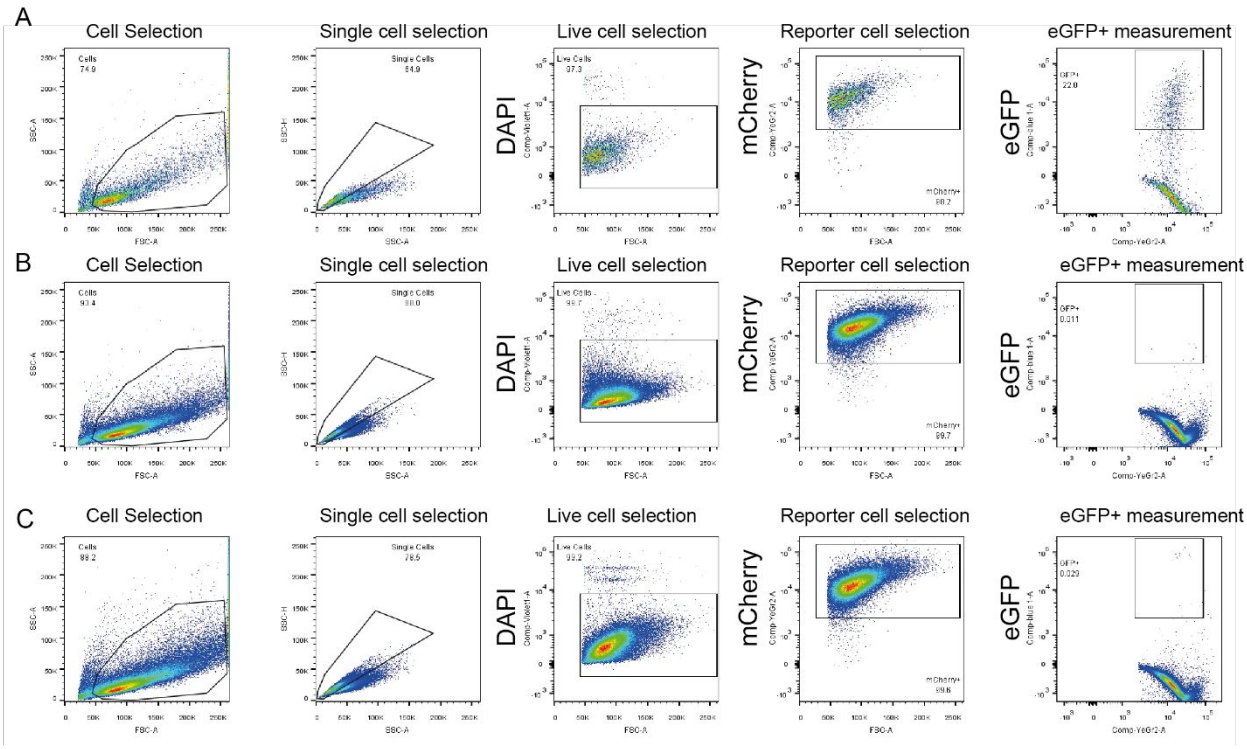


**Figure S4 - Flow cytometry gating strategy used to measure CROSS-FIRE reporter cell activation.**

Firstly, cells are selected by gating using forward scatter area (FSC-A) and sideward scatter area (SSC-A). Singlets are then selected from a SSC-A vs sideward scatter height (SSC-H) plot. Dead cells which can contribute to background signal are removed from the analysis by selecting DAPI negative live cells using their FSC-A and DAPI signals. Reporter cells are then selected using their FSC-A and mCherry signals. From this population, reporter activation is determined by measuring eGFP signal on a mCherry vs eGFP plot. Gates for eGFP+mCherry+ cells were determined using targeting sgRNA transfected positive control cells. (A). For comparison, the same plots are shown for a PBS treated negative control (B). A representative flow cytometry plot of CROSS-FIRE reporter cells treated with targeting sgRNA+ A431-EVs on 6 consecutive days is shown (C).

**Table S1- RNA Sequences**

| | |
|---|---|
| Targeting sgRNA | GGACAGTACTCCGCTCGAGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT |
| Non-targeting sgRNA | TCTCTATCACTGATAGGGAGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT |
| Scaffold sgRNA | UCUCUACCAGGGCUAUGGGCGUUUUAGAGCUAGAAAUAGCAUGUUAAAAUAAUUCUAGUAAGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUUUU |

**Table S2- qPCR Primer Sequences**

| | |
|---|---|
| Targeting sgRNA forward | CAGTACTCCGCTCGAGTGTT |
| Non-targeting sgRNA spike-in forward | TCACTGATAGGGAGGTTTTAGAGC |
| Targeting and non-targeting sgRNA reverse | GACTCGGTGCCACTTTTTCAA |

*References*


1. de Jong OG, Murphy DE, Mäger I, et al. A CRISPR-Cas9-based reporter system for single-cell detection of extracellular vesicle-mediated functional transfer of RNA. *Nat Commun*. 2020;11(1):1113. doi:10.1038/s41467-020-14977-8